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(54) Title: ANTIBODIES FOR THE DETECTION OF HLA-G

(57) Abstract

The Class I molecule HLA-G is specifically expressed by invasive cytotrophoblasts. Antigens which elicit antibodies specific for HLA-G, and antibodies which bind these antigens are provided. Methods related to the measurement of HLA-G levels in biological fluids are also provided.

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ANTIBODIES FOR THE DETECTION OF HLA-G

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BACKGROUND OF THE INVENTION

A central question in pregnancy is how the fetal-placental unit avoids maternal immune rejection. Although fetal and maternal cells interact throughout pregnancy, the fetus typically remains a privileged site, not subject to rejection. It is likely that the particular nature of the cells at the fetal-maternal interface and their products help prevent rejection of the fetus by the maternal immune system.

Implantation and placental development physically connect the mammalian embryo to the maternal uterus. Establishing this connection is essential for subsequent development. The initial developmental events which occur in the embryo set aside unique extraembryonic cellular lineages which are the precursors of the placenta. The first differentiation event gives rise to trophoblasts, which are specialized epithelial cells of the placenta that physically connect the embryo and the uterus (*See*, Cross *et al.* (1994), *Science* 266: 1508 for a comprehensive review of the events surrounding implantation and formation of the placenta).

After fertilization in the oviduct, a series of cell divisions create a mass of totipotent cells (the morula). The first differentiation event occurs after compaction of the morula, leading to formation of the blastocyst. Cells of the trophoblast lineage are formed based upon their position in the morula in a complex cascade of inter- and intra-cell signaling events. In primates, implantation of the blastocyst occurs shortly after the blastocyst hatches from the zona pellucida.

The uterus is made receptive to implantation as a result of events controll d largely by production of estrogen and progesterone from the ovaries. During implantation, trophoblasts attach to the receptive uterine epithelium initiating several changes in the endometrium. Vascular changes occur, such as increased permeability of uterine blood vessels, and inflammatory cells are recruited to the implantation site. Proinflammatory cytokines are produced in the uterus and several cellular changes occur. For example, the uterine epithelium is lost and decidual cells undergo an epithelioid transition and proliferate, producing a massively thickened uterine wall. The decidua also contains abundant macrophages, lymphocytes and other bone-marrow derived cells with unusual properties such as reduced alloreactivity, and responsiveness to stimulation by CD3 antibody.

After implantation in humans, distinct populations of differentiated trophoblasts form. Proliferative cytotrophoblast stem cells are anchored to basement membranes surrounding a stromal core in two types of chorionic villi. In floating villi, cytotrophoblast stem cells detach from the underlying basement membrane and fuse to form a syncytium (a polynucleate cell) which covers the villus and is in direct contact with maternal blood. In anchoring villi, cytotrophoblast stem cells differentiate by detaching from their basement membrane and aggregating to form columns of mononuclear cells which attach to and invade the uterine decidua (interstitial invasion) and its arterial system (endovascular invasion). Interstitial invasion puts cytotrophoblasts in direct contact with the highly specialized subset of leukocytes that home to the uterus during pregnancy. Endovascular invasion puts cytotrophoblasts (like the syncytiotrophoblasts covering the anchoring villi) in direct contact with maternal blood. Thus, antigen presentation by trophoblasts at the maternal-fetal interface is an important component of maternal immunological responses during pregnancy.

MHC class I molecules and the peptides they present regulate alloreactivity (Sherman, *et al.* (1993), *Annu. Rev. Immunol.* 11: 385). Thus, one key to understanding maternal tolerance of the fetal semi-allograft lies in studying trophoblast expression of class I molecules. The molecule HLA-G, which is expressed by placental cells, was cloned in a search for novel class I genes encoded by the human MHC (Geraghty *et al.* (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84: 9145). The gene has an intron/exon organization identical to that of the class Ia genes (HLA-A, -B and -C), and the HLA-G protein product has 86% sequence identity to the class I consensus sequence (Parham *et al.* (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85: 4005). HLA-G has a lower molecular mass (37-39 kDa) than class Ia molecules due to a stop codon in exon 6 that results in the deletion of all but 6 amino acids in the cytoplasmic tail (Shimizu *et al.* (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85: 227). With regard to the 5' flanking region of the gene, the HLA-G promoter has elements (*e.g.*, AP-1, NFκB) similar to sequences found in class Ia genes, but lacks an interferon response element, suggesting novel transcriptional regulatory mechanisms. The primary HLA-G RNA transcript is also differentially spliced; in addition to the full length mRNA, transcripts are produced that lack either exon two, both exons two and three (Ishitani and Geraghty (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89: 3947), or exon four (Kirszenbaum *et al.* (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91: 4209). To what extent these alternatively spliced mRNAs are translated is unclear. Recently, a soluble form of HLA-G encoded by an mRNA containing intron 4 was described (Fujii *et al.* (1994) *J. Immunol.* 153: 5516).

Whereas HLA-A, -B and -C are highly polymorphic, HLA-G appears to exhibit relatively less polymorphism. Immunoprecipitation of HLA-G from 13 individuals and a human choriocarcinoma (malignant trophoblast) cell line showed identical two-dimensional electrophoretic profiles, suggesting reduced polymorphism at this locus. Genomic and

cDNA sequence data also indicate that HLA-G has relatively limited polymorphism. However, a recent study suggests that at least in some populations (*i.e.,* African Americans), HLA-G exhibits substantial polymorphism (van der Ven and Ober (1994) *J. Immunol.* 153: 5628). Whether HLA-G is complexed with endogenous trophoblast peptides and how this repertoire is affected by its degree of polymorphism remains to be determined.

HLA-G is not generally expressed in non-pregnant adults, making it a suitable marker for the diagnosis of pregnancy. The present invention provides antigens for generating specific antibodies to both soluble and membrane bound HLA-G, as well as exemplar antibodies. In addition, HLA-G levels in the maternal blood are indicative of the vigor of cytotrophoblast invasion and the corresponding health of the placental-maternal interface.

Because HLA-G is not generally expressed in adults, it is an ideal marker for diagnosing and monitoring pregnancy and for detecting cytotrophoblasts from biological fluids. However, designing and obtaining suitable antibodies to HLA-G was not previously feasible, due to the high similarity of HLA-G to class Ia molecules which are expressed in adults. This invention overcomes these problems by providing specific epitopes for generating HLA-G-specific antibodies, exemplar antibodies, and methods for their use.

SUMMARY OF THE INVENTION

The present invention provides polypeptides which can be used to elicit antibodies which specifically bind to HLA-G. These antibodies are used, for example, to monitor soluble HLA-G levels in maternal blood. Soluble HLA-G levels in the maternal blood can be used to diagnose pregnancy, or to monitor the health of the fetal maternal interfac .

Accordingly, this invention provides purified polypeptides, comprising a sequence of at least 5 contiguous amino acids selected from an amino acid sequence consisting essentially of amino acid residues 61 to 83 of the α1 domain of the human HLA-G protein, wherein said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to HLA-G, and wherein said peptide does not bind to antisera raised against HLA-G which has been fully immunosorbed with the peptide of Seq. Id. No. 1. An exemplar polypeptide is the polypeptide which consists or essentially of the sequence EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1). These polypeptides are useful as components of an immunogenic composition. In one class of embodiments, the peptide(s) are covalently linked to additional polypeptides such as an immunogenic carrier (*e.g.*, keyhole limpet hemocyanin).

The present invention provides nucleic acids encoding the polypeptides described above which comprise a sequence of at least 5 contiguous amino acids selected from an amino acid sequence consisting essentially of amino acid residues 61 to 83 of the α1 domain of the human HLA-G protein, wherein said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to HLA-G, and wherein said peptide does not bind to antisera raised against HLA-G which has been fully immunosorbed with the peptide of Seq. Id. No. 1. For instance, in one preferred embodiment, the present invention encodes a polypeptide substantially identical to the sequence EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1).

Antibodies which specifically bind to a subsequence in the α1 domain of HLA-G, and methods for making these antibodies are provided. In one class of embodiments, the antibodies specifically bind to subsequences of the amino acid sequence EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1). Exemplar antibodies

include 1B8 and 3F6, described herein. Monoclonal as well as polyclonal antibodies are provided.

Recombinant cells which include nucleic acids encoding the polypeptides and antibodies described above are also provided. Exemplar cell lines include 1B8 and 3F6.

A variety of detection formats are also appropriate, including ELISA, RIA, western blot and other immunoassays.

The invention further provides for kits comprising the various elements described above.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplar immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids

primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist *e.g.,* as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'$_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'$_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the F(ab)'$_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see, Fundamental Immunology*, Third Edition, W.E. Paul, ed., Raven Press, N.Y. (1993), which is incorporated herein by reference, for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies.

The term "biological sample or fluid" refers to material derived from a living organism, including, *e.g.,* blood, cervicovaginal secretions, amniotic fluid, cord blood, urine, tissues, bones and cells.

The term "blood sample" as used herein includes whole blood or derivatives of whole blood well known to those of skill in the art. Thus a blood sample includes the various fractionated forms of blood such as plasma or serum and whole or fractionated blood which additionally comprises various diluents as may be added to facilitate storage or processing in a particular assay. Such diluents are well

known to those of skill in the art and include various buffers, anticoagulants, preservatives and the like.

The term "HLA-G" refers to human leukocyte antigen G and unless otherwise stated includes both the soluble and insoluble forms. The term may in appropriate context refer to either the antigen or the genetic locus.

The term "immunoassay" is an assay that utilizes an antibody to specifically bind an analyte. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, or quantify the analyte.

The terms "isolated" "purified" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

The term "nucleic acid probe" refers to a molecule which binds to a specific sequence or subsequence of a nucleic acid. A probe is preferably a nucleic acid which binds through complementary base pairing to the full sequence or to a subsequence of a target nucleic acid. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labelled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labelled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid r sidues. The terms apply to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (*e.g.,* as commonly used in an ELISA), biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available (*e.g.,* the peptide EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1) can be made detectible, *e.g.,* by incorporating a radiolabel into the peptide, and used to detect antibodies specifically reactive with the peptide).

A "labeled nucleic acid probe" is a nucleic acid probe that is bound, either covalently, through a linker, or through ionic, van der Waals or hydrogen bonds to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

The term "recombinant" when used with reference to a cell indicates that the cell contains nucleic acid with an origin exogenous to the cell. Thus, for example, recombinant cells replicate and/or express genes that are not found within the native (non-recombinant) form of the cell.

The term "identical" in the context of two nucleic acid or polypeptide sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. Optimal alignment of sequences for comparison can be conducted, *e.g.,* by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of

Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection. These references are incorporated herein by reference.

The term "substantial identity" or "substantial similarity" in the context of a polypeptide indicates that a polypeptide comprises a sequence with at least 80% sequence identity to a reference sequence, or preferably 90%, or more preferably 95% sequence identity to the reference sequence, over a comparison window of about 20 amino acid residues. An indication that two polypeptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a polypeptide is substantially identical to a second polypeptide where the two peptides differ only by a conservative substitution.

An indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence dependent and will be different with different environmental parameters. Generally, stringent conditions are selected to be about 5° C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. However, nucleic acids which do not hybridize to each other under stringent conditions are still substantially

id ntical if the polypeptides which they encode are substantially identical. This may occur, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

The phrases "specifically binds to" or "specifically hybridizes to" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind preferentially to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. For example, antibodies can be raised to the peptide EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1) which specifically bind to proteins comprising the sequence EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1) (such as HLA-G) and not to other proteins present in a blood sample. A variety of immunoassay formats are appropriate for selecting antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

Note Regarding Nomenclature: A particular antibody and the cell which produces the antibody are often referred to by the same designation. For instance, the monoclonal antibody 1B8 is produced by the immortalized cell line 1B8.

DETAILED DESCRIPTION

Human placental trophoblasts lie at the maternal-f tal interface, mediating maternal tolerance of the fetus. Central to this mediation is their unusual MHC class I expression; they suppress class Ia production while expressing HLA-G, a class Ib molecule.

A synthetic peptide corresponding to the region from amino acids 61 to 83 of the α1 domain of the HLA-G protein was used to produce monoclonal antibodies which specifically bound HLA-G. The epitope has the advantage of being a linear epitope rather than a conformational epitope. It is available to the antibody without denaturation. It is highly antigenic and produces high titer, high avidity antibodies that detect all forms of HLA-G. Prior to the present invention, there was no way of knowing whether the synthetic peptide would be specific to HLA-G, or whether the conformation of native HLA-G would permit antibody binding to this region. Thus, prior to the present invention, there was no way to know whether antibodies to the synthetic peptide could be generated, or whether any antibodies which were generated would bind to HLA-G. The present invention demonstrates that antibodies are generated to the peptide corresponding to the region from amino acids 61 to 83 of the α1 domain of HLA-G, and that these antibodies specifically bind to HLA-G.

Antibody specificity was demonstrated by immunoaffinity purification of HLA-G from choriocarcinoma cells. These antibodies were incubated with tissue sections of the maternal-fetal interface containing cytotrophoblasts in all stages of differentiation, demonstrating that HLA-G is expressed only by cytotrophoblasts which invade the uterus. *In vitro* studies presented herein show that when early-gestation cytotrophoblast stem cells are cultured they differentiate rapidly along the invasive pathway, upregulating HLA-G production.

Cytotrophoblasts from term placentas, which have reduced invasive capacity *in vitro*, also had decreased ability to upregulate HLA-G

protein expression *in vitro*. High levels of HLA-G mRNA were detected in cytotrophoblasts isolated from first and second trimester placentas that had high invasive capacity. In comparison, term cells contained a greatly reduced level of HLA-G mRNA. Taken together, these results show that HLA-G production is a component of cytotrophoblast differentiation along the invasive pathway.

This invention provides methods and compositions for the generation and use of antibodies which specifically recognize HLA-G. These antibodies were used to measure trophoblast HLA-G expression *in vivo* and *in vitro*. The results showed that HLA-G production is upregulated as an integral part of cytotrophoblast differentiation.

General Techniques

Cloning, PCR, LCR, TAS, 3SR, And QB Amplification

The present invention can be used in conjunction with other techniques such as PCR, TAS, 3SR, QB amplification and cloning, to amplify a any nucleic acid in a biological sample. The nucleic acids of the present invention encode the region of the α1 domain of HLA-G which is specific to HLA-G, *i.e.*, that region of the α1 domain which exhibits the least similarity to other class I molecules. In preferred embodiments, the flanking regions of nucleic acid which encodes amino acid residues 61 to 83 of the α1 domain of the HLA-G protein can be used as a primer binding site for amplification. The products can be used in combination with conventional expression systems to generate the peptide of Seq. ID. No. 1 for purposes of obtaining antigen for antibody production.

More particularly, the nucleic acids of the present invention may be cloned, or amplified by *in vitro* methods, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (3SR) and the Qβ replicase amplification system (QB).

A wide variety of *in vitro* amplification methodologies, cloning and expression systems are known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises may be found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook *et al.*); *Current Protocols in Molecular Biology*, F.M. Ausubel et al., eds. (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel); Cashion et al., U.S. patent number 5,017,478; and Carr, European Patent No. 0,246,864. Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods may be found in Berger, Sambrook, and Ausubel, as well as Mullis et al., (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis et al. eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3, 81-94; (Kwoh, et al. (1989) *Proc. Natl. Acad. Sci. USA* 86, 1173; Guatelli et al. (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell et al. (1989) *J. Clin. Chem* 35, 1826; Landegren et al., (1988) *Science* 241, 1077-1080; Van Brunt (1990) *Biotechnology* 8, 291-294; Wu and Wallace, (1989) *Gene* 4, 560, and Barringer et al.,(1990) *Gene* 89, 117.

Synthetic or chemical means to produce the peptides of Seq. ID. No. 1 are also well known. Automatic peptide synthesizers are available commercially from a variety of sources.

Antibodies to HLA-G

Antibodies may be raised to the polypeptides of the present invention including individual, allelic, strain, or species variants, and

fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Additionally, antibodies can be raised to these polypeptides in either their native configurations or in non-native configurations. Anti-idiotypic antibodies may also be used. Many methods of making antibodies are known to persons of skill. The following discussion is presented as a general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods are known.

a. *Antibody Production*

A number of immunogens may be used to produce antibodies specifically reactive with HLA-G polypeptides. Recombinant or synthetic polypeptides comprising the region from amino acid residues 61 to 83 of the α1 domain of the human HLA-G protein (EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1)) are the preferred polypeptide immunogen for the production of monoclonal or polyclonal antibodies. However, the entire HLA-G moiety, or a subsequence thereof (especially the α1 domain of HLA-G; *see,* Geraghty *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84: 9145) may be used as an antigen, and the resulting antibodies may be screened for HLA-G specificity in the various competitive and non-competitive binding assays described herein, particularly using antibodies generated against the peptid EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1) as competitors. Naturally occurring polypeptides may also be used either in pure or impure form.

Polypeptides are expressed in eukaryotic or prokaryotic cells or chemically synthesized and purified using standard techniques. The polypeptide, or a synthetic version thereof, is injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated for subsequent use in immunoassays to measure the presence and quantity of the polypeptide.

Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen, preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (*e.g.*, keyhole limpet hemanocyanin), or a polypeptide incorporated into an immunization vector such as a recombinant vaccinia virus (*see*, U.S. Patent No. 4,722,848) is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired. *See, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY, which are incorporated herein by reference, and the examples below.

Antibodies, including binding fragments and single chain recombinant versions thereof, against predetermined fragments of polypeptides can be raised by immunization of animals with conjugates of the fragments with carrier proteins as described above. Typically, the immunogen of interest is a peptide of at least about 3 amino acids, and more typically the peptide is 5 amino acids in length or greater. The peptides are typically coupled to a carrier protein, or are recombinantly expressed in an immunization vector. Antigenic determinants on peptides to which antibodies bind are typically 3 to 10 amino acids in length.

Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or modified polypeptides, or screened for agonistic or antagonistic activity, *e.g.*, activity mediated through membrane-bound

HLA-G. Specific monoclonal and polyclonal antibodies will usually bind with a K_D of at least about 500 μM, and most preferably at least about 1 μM or better.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, etc. Description of techniques for preparing such monoclonal antibodies may be found in, *e.g.*, Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *Supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497. Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells are taken from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. The polypeptides and antibodies of the present invention are used with or without modification, and include chimeric antibodies such as humanized murine antibodies.

Other suitable techniques involve selection of libraries of recombinant antibodies in phage or similar v ctors. See, Huse *et al.* (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," *Science* 246: 1275-1281; and Ward, *et al.* (1989) *Nature* 341: 544-546.

Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al.* (1989) *Proc. Nat'l Acad. Sci. USA* 86: 10029-10033.

The antibodies of this invention can also be used for affinity chromatography to isolate HLA-G polypeptides. Columns can be prepared, *e.g.*, with the antibodies linked to a solid support, *e.g.*, particles, such as agarose, Sephadex, or the like, where a cell lysate is passed through the column, washed, and treated with increasing concentrations of a mild denaturant, whereby purified HLA-G polypeptides are released.

The antibodies can be used to screen expression libraries for particular expression products such as HLA-G or for histology studies to locate HLA-G expressing cells. Usually the antibodies in such a procedure will be labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

b. *Immunoassays*

Concentration of HLA-G in a biological sample can be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see Stites and Terr (eds.) 1991 *Basic and Clinical Immunology* (7th ed.). Furthermore, the immunoassays of the present invention can be performed in any of several configurations, *e.g.,* those reviewed in Maggio (ed.) (1980) *Enzyme Immunoassay* CRC Press, Boca Raton, Florida; Tijan (1985) "Practice and Theory of Enzyme Immunoassays," *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers B.V., Amsterdam; Harlow and Lane, *supra*; Chan (ed.) (1987) *Immunoassay: A Practical Guide* Academic Press, Orlando, FL; Price and Newman (eds.) (1991) *Principles and Practice of Immunoassays* Stockton Press, NY; and Ngo (ed.) (1988) *Non-isotopic Immunoassays* Plenum Press, NY.

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte. The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled HLA-G peptide or a labeled anti-HLA-G antibody. Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/HLA-G complex, or to a modified capture group (*e.g.,* biotin) which is covalently linked to the HLA-G peptide or anti-HLA-G antibody.

In a preferred embodiment, the labeling agent is an antibody that specifically binds to the capture agent (anti-HLA-G). Such agents are well known to those of skill in the art, and most typically comprise labeled antibodies that specifically bind antibodies of the particular animal species from which the capture agent is derived. Thus, for example, where the capture agent is a mouse derived anti-human HLA-G

antibody, the label agent may be a goat anti-mouse IgG, *i.e.*, an antibody specific to the constant region of the mouse antibody.

Other proteins capable of specifically binding immunoglobulin constant regions, such as streptococcal protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species. See, generally Kronval, *et al.*, *J. Immunol.*, 111:1401-1406 (1973), and Akerstrom, *et al.*, *J. Immunol.*, 135:2589-2542 (1985).

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 5°C to 45°C.

(i) Non-Competitive Assay Formats

Immunoassays for detecting HLA-G may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case HLA-G) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (anti-HLA-G antibodies) can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture HLA-G present in the test sample. The HLA-G thus immobilized is then bound by a labeling agent, such as a second HLA-G antibody bearing a label. Alternatively, the second HLA-G antibody may lack a label, but it may, in turn, be bound by a labeled third antibody

specific to antibodies of the species from which the second antibody is derived.

Sandwich assays for detecting and/or quantitating HLA-G may be constructed. As described above, immobilized anti-HLA-G specifically binds to HLA-G present in the sample via the epitope defined by Seq. ID. No. 1. Then the second labeled anti-HLA-G (not necessarily HLA-G specific) binds to the bound HLA-G molecule. Free labeled antibody is then washed away and the remaining bound labeled anti-HLA-G is detected (*e.g.*, using a gamma detector where the label is radioactive).

(ii) Competitive Assay Formats

In competitive assays, the amount of analyte (HLA-G) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte displaced (or competed away) from a capture agent (anti HLA-G antibody) by the analyte present in the sample. In one competitive assay, a known amount of analyte is added to the sample and the sample is contacted with a capture agent, in this case an antibody that specifically binds the analyte. The amount of analyte bound to the antibody is inversely proportional to the concentration of analyte present in the sample.

In a particularly preferred embodiment, the capture agent is immobilized on a solid substrate. The amount of HLA-G bound to the capture agent may be determined either by measuring the amount of HLA-G present in an HLA-G/antibody complex, or alternatively by measuring the amount of remaining uncomplexed HLA-G. The amount of HLA-G may be detected by providing a labeled HLA-G.

A hapten inhibition assay is another preferred competitive assay. In this assay a known analyte, in this case HLA-G is immobilized on a solid substrate. A known amount of anti-HLA-G antibody is added to the sample, and the sample is then contacted with the immobilized

HLA-G. In this case, the amount of anti-HLA-G antibody bound to the immobilized HLA-G is proportional to the amount of HLA-G present in the sample. Again the amount of immobilized antibody may be measured by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

Assays for HLA-G Peptides

HLA-G peptides may be defined by their binding properties to antibodies and antisera. As described in the definitions section herein, one indication that two peptides are substantially similar is that they both specifically bind to the same antibody or antibodies. Conversely, if peptides are substantially similar, one peptide is not specifically bound by an antibody which does not specifically bind the second peptide. Thus, the peptides of the present invention can be defined by their binding properties in the various immunoassays described herein. For instance, antisera raised against HLA-G which is immunosorbed with the peptide of Seq. Id. No. 1 (EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1)) until the antisera no longer binds to the peptide of Seq. Id. No. 1 will not react with a peptide which is substantially identical to the peptide of Seq. Id. No. 1. Antisera which has been immunosorbed with the peptide of Seq. Id. No. 1 until it no longer binds specifically to the peptide of Seq. Id. No. 1 is said to be "fully immunosorbed."

In order to produce antisera for use in these immunoassays, HLA-G is used to immunize mice from an inbred strain, such as BALB/c, using a standard adjuvant, such as Freund's adjuvant, and a standard mouse immunization protocol (see Harlow and Lane, *supra* and the procedures described herein). Alternatively, a synthetic peptide derived

from HLA-G and conjugated to a carrier protein or a peptide vector containing th sequence can be used an immunogen. Polyclonal sera are collected and titered against the immunogen protein in an immunoassay as described herein, for example, a solid phase immunoassay with the immunogen (*e.g.,* HLA-G synthetic peptide) immobilized on a solid support. Polyclonal antisera with a titer of 10^4 or greater are selected and tested for their ability to bind HLA-G, using a competitive binding immunoassay as described above, *e.g.,* using generic competitors which are unrelated to HLA-G (*e.g.,* bovine serum albumin). Antisera which bind HLA-G are then selected for characterization of a peptide of interest, *i.e.,* one which may be substantially identical to the peptide of SEQ Id. No. 1.

Immunoassays in the competitive binding format are then used for cross-reactivity determinations between the peptide of Seq. Id. No. 1 and a target peptide. For example, the protein of Seq Id No. 1 can be immobilized to a solid support and used to isolate antibodies which specifically bind to the peptide of Seq. Id. No. 1 from the antisera. The ability of a target protein to bind the antibodies isolated from the pooled antisera, and to the pooled antisera which has been stripped of antibodies which bind to the peptide of Seq. Id. No. 1 is then compared to that observed for the peptide of Seq. Id. No. 1. Where the target polypeptide and the peptide of sequence Id. No. 1 have an affinity for both the immunoabsorbed polyclonal antisera (the antisera which does not specifically bind to the peptide of Seq. Id. No. 1) and the antibodies isolated during the immunoabsorbtion process (the antisera which does bind the peptide of Seq. Id. No. 1) which is the same within the experimental error of the system, plus or minus 10%, the polypeptides are substantially identical. The experimental error of the system is monitored by using identical polypeptides as controls from one experiment to the next, *i.e.,* identical polypeptides have the same

antibody binding properties, and any discrepanci s reflect the xperimental error in the system.

Assays for Soluble HLA-G

A. *Sample Collection and Processing*

Soluble HLA-G is preferably quantified in a biological sample derived from a patient. Particularly preferred biological samples include blood and cervicovaginal secretions. U.S. Patent No. 5,096,830 describes cervicovaginal secretions as diagnostic assay samples, and provides means for taking such samples.

In a preferred embodiment, HLA-G is quantified in whole blood or blood derivatives such as blood plasma or blood serum. Blood samples are isolated from a patient according to standard methods well known to those of skill in the art, most typically by venipuncture.

The sample may be pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used.

B. *Quantification of HLA-G.*

HLA-G may be detected and quantified by any of a number of means well known to those of skill in the art. These include analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like.

Where levels of soluble HLA-G in the biological sample (*e.g.,* sera) are low (*e.g.,* 1-100 nM) radio immunoassays (RIAs) and capillary electrophoresis are preferred methods of monitoring the level of soluble HLA-G. In one class of embodiments, the biological sample is enriched for soluble HLA-G, *e.g.,* by removing non-HLA-G components prior to performing the assay. It is often desirable to fractionate the biological sample prior to performing the above-described techniques to increase the sensitivity of any of the assays above for the detection of soluble HLA-G. For instance, an initial rough separation of soluble HLA-G from other biological components can be performed by appropriate centrifugation, filtration, column chromatography, or isotonic washing of the biological sample. In addition, certain non-HLA-G components of the biological sample can be specifically removed from the sample using the techniques described herein, where capture agents specific for non-HLA-G components are used to remove those non-HLA-G components from the sample. For instance, erythrocytes can be specifically removed from a biological sample comprising blood by, *e.g,* immunoadsorbtion or affinity chromatography.

C. *Reduction of Non-Specific Binding*

One of skill in the art will appreciate that it is often desirable to reduce non-specific binding in immunoassays and analyte purification. Where the assay involves an antigen, antibody, or other capture agent immobilized on a solid substrate, it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used.

D. *Other Assay Formats*

Western blot analysis can also be used to detect and quantify the presence of HLA-G in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind HLA-G. The anti-HLA-G antibodies specifically bind to HLA-G on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-mouse antibodies where the antibody to HLA-G is a murine antibody) that specifically bind to the anti-HLA-G.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (see, Monroe *et al.*, *Amer. Clin. Prod. Rev.* 5:34-41 (1986)), which is incorporated herein by reference.

E. *Labels*

The particular label or detectable group used in the assay is not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, most labels useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention includ magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein

isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horseradish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation of the compound, stability requirements, available instrumentation, and disposal provisions.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labelling or signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by reference.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence, *e.g.*, by microscopy, visual inspection, via photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally, simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

F. *Substrates*

As mentioned above, depending upon the assay, various components, including the antigen, target antibody, or anti-idiotypic antibody, may be bound to a solid surface. Many methods for immobilizing biomolecules to a variety of solid surfaces are known in the art. For instance, the solid surface may be a membrane (*e.g.*, nitrocellulose), a microtiter dish (*e.g.*, PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (*e.g.* glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass, silica, plastic, m tallic or polymer bead. The desired

component may be covalently bound, or noncovalently attached through nonspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, are included substances that form gels, such as proteins (*e.g.*, gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, *e.g.*, as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. See, for example, *Immobilized Enzymes*, Ichiro Chibata,

Halsted Press, New York, 1978, and Cuatrecasas, *J. Biol. Chem.* 245 3059 (1970) which are incorporated herein by reference.

In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labeled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082, which are incorporated herein by reference.

G. *Comparison of HLA-G Levels to a Sample Population*

In one class of preferred embodiments, the assays of the present invention are used to quantify average soluble HLA-G levels in a reference population of pregnant women for comparison to, *e.g.*, a particular pregnant patient. Any of the techniques described herein for quantitating an analyte are used to quantify HLA-G levels in biological samples from a particular population such that an average level of HLA-G in, *e.g.*, the maternal blood is derived for the population. The population is selected such that all members of the population are in the same term of their pregnancy, are all apparently healthy with apparently normal pregnancies, and such that all members of the population are of a similar genetic background (*i.e.*, all members of a single racial sub-type, *e.g.*, Northern Europeans). This population is termed a "reference population." Individual patients with the same general genetic background and in the same gestational age of their pregnancy are then

compared to the reference population to determine whether their HLA-G levels are normal (*i.e.,* the same as the reference population).

Determination of HLA-G Levels for Monitoring Pregnancy

Because adults do not express HLA-G, the presence of HLA-G, *e.g.,* in the blood indicates that a patient is pregnant. The presence of HLA-G in maternal blood may be determined by the methods described above. The level of HLA-G in the maternal blood may be a general indicator of the health of the fetal-maternal interface. Because HLA-G is expressed largely by invasive trophoblasts, an elevated or reduced level of HLA-G in the maternal blood of a patient compared to a reference population could indicate that trophoblast invasion is proceeding abnormally.

The present invention also provides kits for the diagnosis of pregnancy and disease states related to abnormal levels of circulating HLA-G levels. The kits preferably include an antibody that specifically binds to HLA-G. The antibody may be free or immobilized on a solid support as described above. The kit may also contain, *e.g.,* instructional materials teaching the use of the antibody in an assay for the detection of pregnancy, appropriate diluents, chemical reagents and the like.

Localization of HLA-G Expression

The interpretation of localization studies to determine the temporal and spatial pattern of placental HLA-G expression was previously complicated by the use of antibodies and probes that react with other class I molecules. Earlier immunolocalization studies describing placental MHC class I expression were performed with monoclonal antibodies that do not distinguish between class Ia and Ib molecules. More recently, investigators localized HLA-G mRNA within the placenta by *in situ* hybridization. All of these studies detect d HLA-G mRNA in extravillus cytotrophoblasts. Two of these investigators

detected HLA-G mRNA in early-gestation villus cytotrophoblasts and villus mesenchyme (Yelavarthi, *et al.* (1991) *J. Immunol.* 146: 2847; and Chumbley, *et al.* (1993) *Hum. Immunol.* 37: 17). In contrast, others reported that the transcript is present only in syncytiotrophoblasts (Lata (1992) *J. Exp. Med.* 175: 1027).

In agreement with the *in situ* hybridization data, the immunolocalization studies presented herein (*see, e.g.*, the Examples section below) show that extravillus cytotrophoblasts express HLA-G protein. However, no reactivity with anti-HLA-G antibodies in villus cytotrophoblasts, syncytiotrophoblasts (except for weak brush border staining in the first trimester) or elements of the villus core were observed. The discrepancy between the presence of the HLA-G mRNA and the HLA-G proteins in specific tissues indicates that HLA-G transcripts are not translated, or alternatively, are spliced and result in a form of the protein not recognized by certain antibodies. However, the amino acid sequence to which these antibodies were raised is not deleted in any of the alternatively spliced mRNAs which have been described.

It is also possible that nucleic acid probes used in previous studies resulted in artifacts from cross reaction of the probes with class Ia mRNAs present in the villus core. Supporting this possibility, northern hybridization using probes corresponding to the 450 bp *Pvu* II fragment from the 3' untranslated region of the HLA-G cDNA cross-reacted with class Ia mRNAs under all but the most stringent conditions (*See*, Examples below).

Purified First and Second Term cytotrophoblasts Up-Regulate HLA-G Expression in vitro

To study dynamic aspects of cytotrophoblast differentiation along the invasive pathway, an *in vitro* model of this process was used. Cytotrophoblast stem cells, isolated from early-gestation placentas,

rapidly invade extracellular matrices on which they are plated. During this process they switch their repertoire of integrin extracellular matrix receptors, (as do cytotrophoblasts invading the uterus *in vivo*) and upregulate 92 kDa type IV collagenase synthesis/ activation (Librach *et al.* (1994) *J. Biol. Chem. 269:17125*). Perturbing either adhesion molecule or proteinase function inhibits invasion, suggesting that these molecules play key roles in acquisition of cytotrophoblast invasiveness. Expression of these molecules is controlled, at least in part, at the translational level; however, upregulation of proteinase and integrin expression *in vitro* is paralleled by an upregulation in production of the corresponding mRNAs. Cytotrophoblast proteinase and integrin expression *in vitro* also varies dramatically as gestation proceeds. Term cytotrophoblasts, which have lost their invasive capacity, produce little of the 92 kDa type IV collagenase, fail to undergo integrin switching and contain no detectable mRNA encoding these molecules.

As with proteinases and integrins, first and second trimester cytotrophoblasts upregulated HLA-G expression in culture. Immunolocalization studies performed on tissue sections showed that villus cytotrophoblasts do not react with anti-HLA-G antibodies. Thus, as would be expected, only a small percentage of isolated villus cells produce HLA-G immediately after plating. After 12 h in culture, 62% of first trimester cytotrophoblasts expressed HLA-G. However, it is clear that cytotrophoblast HLA-G expression is regulated differently from that of integrins and proteinases. HLA-G mRNA levels remained constant throughout the culture period. There are two possible explanations for this phenomenon. First, cytotrophoblast stem cells may contain high levels of HLA-G mRNA; isolating them does not change these levels, but promotes translation. Second, HLA-G mRNA production could be induced to maximal levels during the isolation procedure. In either case, it is clear that the time course of cytotrophoblast HLA-G mRNA production *in vitro* differs from that of proteinases and adhesion

molecules. Surprisingly, a significant percentage (42%) of term cells, which no longer express antigens involved in invasion, upregulated HLA-G expression in culture even though they contained much lower HLA-G mRNA levels than early gestation cells.

Together these observations reflect the fact that the placenta performs many unique functions, some of which change dramatically during pregnancy. Enhanced metalloproteinase expression and integrin switching are characteristics of the early gestation cytotrophoblasts that mediate uterine invasion. Downregulating the expression of these genes is one mechanism that controls cytotrophoblast invasiveness. In contrast, extravillus cytotrophoblasts of all gestational ages retain the ability to upregulate HLA-G expression *in vitro*. These observations are consistent with the fact that the cells need only transiently express invasive characteristics, while consistently avoiding maternal immune surveillance.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially similar results.

Example 1: Characterization of the anti-HLA-G monoclonal antibodies

Monoclonal antibodies to HLA-G were generated by immunizing mice with a synthetic peptide corresponding to amino acids 61-83 of the α1 domain of HLA-G (EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1)).

The peptide was coupled to maleimide-activated keyhole limpet hemocyanin (KLH; Imject; Pierce Chemical Co., Rockford, IL) via a C-terminal cysteine (added for this purpose) according to the

manufacturer's instructions. Two-month-old female BALB/c mice (Charles River) were given i.p. injections of 100 μg peptide-KLH conjugate emulsified in Freund's complete adjuvant and were boosted after 14 days with 50 μg antigen in incomplete adjuvant. Test bleeds were obtained 10 days later from tail veins, and sera were screened by ELISA (described below) for reactivity against the peptide immunogen. Mice exhibiting the best humoral responses were given a final intravenous boost (50 μg) and sacrificed three days later. Spleen cells were isolated and fused with SP2/0 myeloma cells according to published procedures (Kohler and Milstein (1975) *Nature* 256: 495; and Harlow and Lane (1988), *Supra*). Cultures were selected in HAT medium (UCSF Cell Culture Facility) and cloned by limiting dilution.

The HLA-G antibodies isolated using the above procedure belonged to the IgM class. At least two factors probably contributed to this outcome. First, the mice were immunized for a relatively short period of time before the hybridomas were produced, and early antigenic responses result primarily in IgM-class antibody production. Second, the reporter antibodies used in the hybridoma screening react with IgM as well as IgG. Using the above method, one can reproducibly obtain HLA-G specific antibody producing hybridomas at at least 1% or greater.

Two methods to determine whether 1B8 antibody reacted with class Ia molecules (HLA-A, -B, -C) were used. Flow cytometry experiments (flow cytometry techniques are described below) indicated that peripheral blood leukocytes isolated from the blood of 12 different individuals did not bind the antibody. In contrast, JEG-3 cells and a B-lymphoblastoid HLA-null cell line (LCL 721.221) stably transfected with a vector expressing HLA-G gave strong positive signals when stained with 1B8 or W6/32, an antibody that recognizes monomorphic determinants of all class I heavy chain/β2-microglobulin complexes (Barnstable *et al.* (1978) *Cell* 14: 9). However, the parental (untransfected) lymphoblastoid cells reacted with neither antibody. An

isotype-matched irrelevant IgM (Sigma) did not react with any of the cells.

That the antibody does not react with class Ia molecules is further supported by our immunolocalization data (discussed in detail in the following sections). The placenta and placental bed contain many cells that express class Ia antigens. For example, a hallmark of human pregnancy is leukocyte infiltration of the decidua (Ferry *et al.* (1990) *Immunology* 70: 446). Floating chorionic villi, composed entirely of fetal cells, contain abundant stromal cells and macrophages (Hofbauer cells) which also express class Ia proteins (Nakamura *et al* (1990) *Hum. Pathol.* 21: 936). However, immunolocalization studies performed on placental tissue from 26 individuals showed that none of these class Ia-expressing cell populations stained with HLA-G antibodies; the 1B8 and 3F6 mAbs reacted only with specific populations of cytotrophoblasts.

Example 2: Antibody screening

Hybridomas were screened for reactivity against the peptide immunogen by antibody-capture ELISA according to standard methods (*see,* Harlow and Lane, *supra*). Microtiter plates were coated with 50 µl of PBS containing 10 µg/ml of the synthetic peptide, washed three times with PBS and blocked for 1 hour at room temperature with PBS containing 0.02% (v/v) Tween 20, 0.25% BSA (w/v) and 0.02% sodium azide (w/v; blocking buffer). Plates were incubated with test fluids (test sera or hybridoma supernatants) for 1 h at room temperature, followed by rabbit anti-mouse IgG conjugated to alkaline phosphatase (Jackson Immuno Research Labs., Inc., West Grove PA) diluted 1:2000 in blocking buffer. Reactivity was assessed by adding 50 µl *p*-nitrophenyl phosphate substrate solution (3 mM PNPP, 0.05 M $NaCO_3$, 0.5 mM $MgCl_2$, pH 9.5) and measuring the absorbance at 405 nm with a microplate reader (Molecular Devices Inc., Menlo Park, CA).

Hybridomas were also screened for their ability to r act with cytotrophoblasts in tissue sections of early second trimester (16-18 week) placentas that contained anchoring villi. Double indirect immunofluorescence was performed using hybridoma supernatants and a rat monoclonal IgG against cytokeratin (7D3). The cytokeratin antigen is present in differentiated epithelial cells, but not on connective tissue, endothelium, muscle or blood cells.

Placental tissues were fixed for 30 min with 3% paraformaldehyde in calcium-containing PBS, pH 7.2. They were then incubated in 10 mM glycine to quench unreacted aldehyde groups, infiltrated with 15% sucrose, embedded in OCT (Miles Scientific, Naperville, IL), and frozen in liquid nitrogen. Sections (5 μm) were cut using a Slee HR cryostat and collected on 22-mm^2 coverslips. Before staining, the sections were washed for 10 min each in PBS and in PBS containing 0.2% BSA. Primary antibodies were applied to the tissue sections as undiluted hybridoma supernatants for 1 h at room temperature. After being washed, sections were incubated for 30 min at room temperature with a 1:1 mixture of fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rat IgG (diluted 1:200; Jackson Immuno Research). The samples were mounted and examined with a Zeiss epifluorescence-phase microscope and photographed with Kodak Tri X film.

The class of the antibodies selected for further characterization was determined using an ImmunoType Kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Several antibodies were isolated that bound HLA-G peptide using the ELISA and stained cytotrophoblasts in the first trimester placenta. Two of these antibodies (1B8 and 3F6), both IgM class antibodies, were further characterized.

Example 3: Affinity purification of HLA-G

To characterize the specificity of the antibodies, we constructed an affinity column using the 1B8 mAb, through which we passed an ^{35}S-labeled JEG-3 detergent extract. This choriocarcinoma cell line expresses HLA-G, but not class Ia molecules. After washing, bound proteins were eluted and analyzed by 10% SDS-PAGE and fluorography. A single 38 kDa protein, corresponding to the molecular mass of the HLA-G α chain, was detected. β2-microglobulin (12 kDa) would be expected to run off the bottom of the gel under the conditions used.

The immunoaffinity column was constructed using the 1B8 antibody as follows. First, the peptide immunogen was coupled to thiopropyl-activated Sepharose 6B (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. Antibody was then bound to the matrix by passing 1B8 ascites fluid, diluted 1:10 in PBS, through the column and washing with 20 bed volumes of PBS. That the antibody was specifically bound was demonstrated by eluting the column with 100 mM glycine, pH 2.5. This fraction contained pure IgM as assessed by silver staining of SDS-polyacrylamide gels.

The antibody column was used to purify HLA-G from ^{35}S-labeled JEG-3 choriocarcinoma cell extracts as follows. 1 x 10^7 JEG-3 cells were metabolically labeled by overnight incubation in methionine- and cysteine-free DMEM (Gibco, Gaithersburg, MD) containing 0.5 mCi/ml ^{35}S-protein labeling mix (EXPRE^{35}S^{35}S Protein Labeling Mix, >1000 Ci/mmol, New England Nuclear, Boston, MA). After labeling, cells were washed once with cold PBS and lysed with cold buffer containing 50 mM Tris and 1% NP-40, pH 8.0. Lysates were cleared by centrifugation at 16,000 x *g* for 10 min at 4° C, and supernatants were applied to the column. The column was washed with 20 bed volumes of PBS and eluted with 100 mM glycine, pH 2.5. Eluates and flow-

through fractions (800 cpm per sample) were separated by 10% SDS-PAGE, processed for fluorography, and exposed to x-ray film at -80°C.

Example 4: Flow cytometry

Peripheral blood lymphocytes (PBLs) were prepared by centrifugation of whole blood through Ficoll-Hypaque 1077 (Sigma). Single-cell suspensions of JEG-3 were obtained by trypsinization of monolayer cultures grown in Eagle's minimum essential medium (UCSF Cell Culture Facility) supplemented with 10% fetal bovine serum (Sigma). PBLs or JEG-3 cells were washed in PBS containing 1 mg/ml BSA and incubated with either 1B8 (hybridoma supernatant diluted 1:10) or W6/32 (1 μg/ml) followed by fluorescein-conjugated anti-mouse IgM or IgG, respectively (diluted 1:100; Jackson Immuno Research). Cells were then washed, fixed in 0.2% paraformaldehyde, and analyzed on a FACScan cytometer (Becton Dickinson, Mountain View, CA).

Example 5: Immunofluorescent localization of HLA-G in the placenta and placental bed; HLA-G is expressed by cytotrophoblasts that differentiate along the invasive pathway in vivo.

Immunohistochemical analysis using fluorescent detection was carried out on frozen sections of placenta and placental bed prepared from tissues obtained during the first, second and third trimesters of pregnancy. Frozen sections were prepared from first, second and third trimester human chorionic villi or placental bed biopsies as previously described (Damsky *et al.* (1992) *J. Clin. Invest.* 89: 210). Double indirect immunofluorescence using 1B8 or 3F6 (anti-HLA-G antibodies) and 7D3 (anti-cytokeratin) was performed essentially as described above. Reactivity of the 1B8 and 3F6 antibodies was detected using fluorescein-conjugated goat anti-mous IgM, diluted 1:200 (Jackson Immuno Research). Control experiments included

incubation of tissues with primary or secondary antibodies alone, non-immune mouse serum or normal mouse IgG.

Sections contained floating chorionic villi and anchoring villi (including cytotrophoblast cell columns), as well as decidualized endometrium and myometrium. Thus, cytotrophoblast stem cells, as well as differentiated trophoblasts (syncytiotrophoblasts and invasive cytotrophoblasts), were evident. Sections were double-stained with an anti-cytokeratin antibody (7D3), which in the placental bed is specific for trophoblast cells, and either the 1B8 or 3F6 anti-HLA-G mAbs (described *supra*).

The 3F6 mAb gave an identical staining pattern to that of 1B8. None of the components of floating villi, including undifferentiated cytotrophoblasts anchored to the villus basement membrane and fetal elements within the villus core, reacted with the anti-HLA-G antibodies. In contrast, invasive cytotrophoblasts within the cell columns of anchoring villi stained brightly (Fig. 2, panel B). Antibody reactivity was first detected in the distal part of the cell columns as the cytotrophoblasts made contact with the uterine wall. Cytotrophoblasts participating in interstitial invasion also stained brightly. During this stage of pregnancy, when endovascular invasion peaks, cytotrophoblasts within blood vessels also showed intense reactivity with the anti-HLA-G antibodies. Incubation of tissues with primary or secondary antibodies alone, non-immune mouse serum or normal mouse IgG showed no reactivity.

Cytotrophoblast staining in first trimester samples was nearly identical to that in second trimester tissue. None of the floating villi components reacted with either antibody. The only exception was occasional syncytial brush border staining. Third trimester tissue exhibited the same pattern; floating villi (including the syncytial brush border) did not stain whereas interstitial and endovascular cytotrophoblasts reacted with the antibodies. However, by the third

trimester, cytotrophoblasts within uterine wall stained less brightly than cells in a comparable location earlier in gestation.

Example 6: HLA-G is expressed by cytotrophoblasts that differentiate along the invasive pathway in vitro

Highly purified early gestation cytotrophoblast stem cells plated on Matrigel aggregate and acquire an invasive phenotype, as shown by the expression of stage-specific antigens. This experimental system was used to examine HLA-G production during the course of cytotrophoblast differentiation along the invasive pathway *in vitro*. Two culture conditions were employed. In the first, early-gestation cytotrophoblasts were plated on Matrigel plugs, which promotes the formation of large aggregates. Staining sections of these aggregates allowed determination of the spatial pattern of HLA-G expression as the cells invaded the extracellular matrix substrate. Cells on the surface that had not invaded the Matrigel did not stain, while those that had penetrated the substrate reacted with the 1B8 antibody.

Highly purified cytotrophoblasts were prepared from first, second and third trimester chorionic villi as previously described (Fisher *et al.* (1989) *J. Cell Biol.* 109: 891; and Librach, *et al.* (1991) *J. Cell Biol.* 113: 437). Cells were plated on the laminin-rich extracellular matrix preparation Matrigel (Collaborative Research, Bedford, MA), in MEM (UCSF Cell Culture Facility) containing 2% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, IN). To promote the formation of large aggregates, 2.5×10^5 cells were plated on plugs of Matrigel formed in capsules (6.5 mm diameter). After 3 days in culture, the Matrigel plugs and cytotrophoblast aggregates were fixed in 3% paraformaldehyde, sectioned and processed for immunostaining. Sections of aggregates that had invaded Matrigel plugs were stained essentially as described above, except that reactivity was detected using

a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

The second culture condition was used to determine the time course of HLA-G protein production *in vitro*. Cytotrophoblasts isolated from placentas of different gestational ages were cultured on a thin layer of Matrigel. Under these conditions the cells differentiate along the invasive pathway, as they do when cultured on Matrigel plugs (Damsky *et al.* (1994) *Development* 120: 3657). However, they form smaller aggregates, which permits determination of the staining pattern of individual cells without sectioning. Cytotrophoblasts of all gestational ages upregulated HLA-G production in culture. For example, immediately after plating approximately 25% of first trimester cells expressed HLA-G, but by 12 h in culture nearly 60% reacted with the antibody. This level remained constant throughout the 48 h assay period. With increasing gestational age there was a decrease in the percentage of immunopositive cells at most time points. For example, significantly fewer third than first trimester cells expressed HLA-G after 12 h (34 % vs. 63 %). The assays were performed as described below.

To determine the percentage of cells expressing HLA-G after various times in culture, 2.5×10^5 cells were plated on coverslips (22 mm^2) coated with a thin layer of Matrigel (10 μl). After 0-48 h of culture, the cells were fixed in 3% paraformaldehyde for 5 min at room temperature, washed with PBS, permeabilized with cold methanol for 5 min and processed for immunofluorescence exactly as described above.

Example 7: Northern hybridization shows that Cytotrophoblast HLA-G mRNA production in vitro is gestationally regulated

Northern hybridization was used to quantify mRNA levels in cultured first, second and third trimester cytotrophoblasts. High levels of HLA-G mRNA were detected in first and second trimester cytotrophoblasts immediately after isolation, and the level of mRNA

expression did not change over time in culture. In comparison, term cells contained a greatly reduced level of HLA-G mRNA throughout the culture period. The northern analysis was performed as described below.

Total RNA was extracted from cultured cytotrophoblasts according to published methods (Chomczynski and Sacchi (1987) *Anal. Biochem.* 162: 156). Briefly, 2-5 x 10^7 cells per sample were homogenized in 500 μl guanidine buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl), after which 50 μl 2 M sodium acetate (pH 4), 500 μl water-saturated phenol and 100 μl chloroform were added. After centrifugation, RNA was pelleted from the aqueous phase by the addition of 500 μl isopropanol, extracted with 4 M LiCl, and reprecipitated from a solution containing 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.5% SDS. The pellets were then washed with 70% ethanol, vacuum dried and dissolved in sterile water. The concentration of RNA was determined by measuring the absorbance at 260 nm.

An HLA-G-specific cDNA probe was synthesized by random priming of the 450 bp *Pvu* II fragment from the 3' untranslated region of HLA-G using ^{32}P-CTP and the Klenow fragment of DNA polymerase I according to standard methods (Tabor *et al.* (1993) In *Current Protocols in Molecular Biology,* vol. 1. K. Jannssen, ed. John Wiley and Sons, New York p. 3.0.1). Probes had a specific activity of 2 x 10^9 dpm/μg. Total RNA (10 μg) was separated by formaldehyde-agarose gel electrophoresis, transferred to Nytran membranes (Schleicher and Schuell, Keene, NH) and analyzed by Northern blot hybridization as previously described (Lehrach *et al.* (1977) *Biochemistry* 16: 4743; and De *et al.* (1990) *J. Biol. Chem.* 265: 15267)). In all experiments, gels were stained with acridine orange prior to transfer to ensure integrity of the RNA samples, and to confirm that equal amounts of RNA had been loaded onto each lane. The final post-hybridization washes were carried

out in 0.3 X SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.4) and 0.1% SDS at 68° C.

All publications and patent applications cited in this specification are herein incorporated by reference for all purposes as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

(ii) TITLE OF INVENTION: ANTIBODIES FOR THE DETECTION OF HLA-G

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 90012-2628

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Berliner, Robert
(B) REGISTRATION NUMBER: 20,121
(C) REFERENCE/DOCKET NUMBER: 5555-372

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: (213) 977-1003

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu Glu Glu Thr Arg Asn Thr Lys Ala His Ala Gln Thr Asp Arg Met
1 5 10 15

Asn Leu Gln Thr Leu Arg Gly
20

WHAT IS CLAIMED IS:

1. A purified polypeptide, comprising a sequence of at least 5 contiguous amino acids selected from an amino acid sequence consisting essentially of amino acid residues 61 to 83 of the α1 domain of the human HLA-G protein, wherein said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to HLA-G, and wherein said peptide does not bind to antisera raised against HLA-G which has been fully immunosorbed with the peptide of Seq. Id. No. 1.

2. The polypeptide of claim 1, comprising the sequence EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1).

3. A polypeptide substantially identical to the polypeptide with the sequence EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1).

4. The polypeptide of claim 3 with the sequence EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1).

5. An immunogenic composition comprising the polypeptide of claim 1 and pharmaceutically acceptable excipients.

6. The immunogenic composition of claim 5, wherein the polypeptide of claim 1 is covalently linked to a second polypeptide.

7. The immunogenic composition of claim 5, wherein the immunogenic composition further comprises keyhole limpet hemocyanin.

8. A nucleic acid encoding a polypeptide comprising a sequence of at least 5 contiguous amino acids selected from an amino acid sequence consisting essentially of amino acid residues 61 to 83 of the α1 domain of the human HLA-G protein, wherein said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to HLA-G, and wherein said peptide does not bind to antisera raised against HLA-G which has been fully immunosorbed with the peptide of Seq. Id. No. 1.

9. The nucleic acid of claim 8, wherein the nucleic acid encodes a polypeptide substantially identical to the sequence EEETRNTKAHAQTDRMNLQTLRG (Seq. Id. No. 1).

10. An antibody which specifically binds to a subsequence in the α1 domain of HLA-G wherein the subsequence is located within the sequence of Seq. ID. No. 1.

11. The antibody of claim 10, wherein the antibody is selected from the group consisting of 1B8 and 3F6.

12. A recombinant cell which comprises a nucleic acid encoding a polypeptide comprising a sequence of at least 5 contiguous amino acids selected from an amino acid sequence consisting essentially of amino acid residues 61 to 83 of the α1 domain of the human HLA-G protein, wherein said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to HLA-G, and wherein said peptide does not bind to antisera raised against HLA-G which has been fully immunosorbed with the peptide of Seq. Id. No. 1.

13. A cell which produces an antibody which specifically binds to Seq. ID. No. 1.

14. The cell of claim 13 selected from the group consisting of the cell lines 1B8 and 3F6.

15. A method for making antibodies which specifically bind to HLA-G, comprising exposing an animal to an immunogenic composition comprising a polypeptide with a sequence of at least 5 contiguous amino acids selected from an amino acid sequence consisting essentially of amino acid residues 61 to 83 of the α1 domain of the human HLA-G protein, wherein said peptide does not bind to antisera raised against HLA-G which has been fully immunosorbed with the peptide of Seq. Id. No. 1.

16. The method of claim 15, wherein the polypeptide consists of the sequence EEETRNTKAHAQTDRMNLQTLRG (Seq. ID. No. 1).

17. The method of claim 15, wherein the method further comprises immortalizing antibody producing cells of the animal exposed to said immunogenic composition, and isolating said immortalized cells.

18. An antibody produced by the method of claim 15.

19. A method of identifying HLA-G in a biological sample comprising the steps of:

i) hybridizing an antibody which specifically binds to a portion of the α1 domain of HLA-G with the biological sample wherein said portion consists of an epitope defined by Seq. ID. No. 1; and

ii) detecting specific binding between the antibody and the biological sample.

20. The method of claim 19, wherein step (ii) comprises detecting the concentration of soluble HLA-G in a biological sample.

21. The method of claim 19, wherein step (ii) comprises detecting the concentration of insoluble HLA-G in a biological sample.

22. The method of claim 19 wherein the biological sample is a female human blood sample.

23. The method of claim 19 wherein the biological sample is a cervicovaginal secretion.

24. The method of claim 19, wherein step (ii) is performed using a radioimmunoassay.

25. The method of claim 19, wherein the antibody is attached to a solid substrate.

26. The method of claim 19, wherein the biological sample is female human blood comprising soluble HLA-G, and wherein the antibody specifically binds to the soluble HLA-G, and wherein the formation of a specific antibody-soluble HLA-G complex is diagnostic of pregnancy.

(27) The method of claim 19, wherein the biological sample is female human blood comprising soluble HLA-G, and wherein the method further comprises quantitating the specific binding of the antibody to the soluble HLA-G in the female human blood.

28. The method of claim 19, wherein the biological sample is female human blood comprising soluble HLA-G, and wherein the method further comprises:

(iii) quantitating the level of specific binding of the antibody to the soluble HLA-G; and

(iv) comparing the level of specific binding to a reference population.

29. A kit for detecting HLA-G which comprises a container containing an antibody specifically binding to a polypeptide consisting of the amino acid sequence of Seq. ID. No. 1.

30. A kit of claim 29 which further comprises a container containing a polypeptide consisting essentially of Seq. ID. No. 1.

31. A kit of claim 29 which further comprises a container containing HLA-G.

INTERNATIONAL SEARCH REPORT

Ir. ...tional Application No
PCT/US 96/03765

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/74 C07K16/28 A61K39/00 C12N5/10
C12N5/20 G01N33/566 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE FASEB JOURNAL, vol. 4, no. 7, 26 April 1990, BETHESDA, MD, USA, page A2216 XP002007027 D. GERAGHTY ET AL.: "Production of monoclonal antibodies specific for the new class I antigen HLA-G and their use to examine expression in trophoblast cells." see abstract 3016 --- -/--	1-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 1996	25.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nooij, F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 89, no. 9, 1 May 1992, WASHINGTON, DC, USA, pages 3947-3951, XP002007028 A. ISHITANI ET AL.: "Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens." see abstract see page 3949, right-hand column, line 18 - page 3950, left-hand column, line 3 ---	1-31
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 84, no. 24, December 1987, WASHINGTON, DC, USA, pages 9145-9149, XP002007029 D. GERAGHTY ET AL.: "A human major histocompatibility complex class I gene that encodes a protein with a shortened cytoplasmic segment." cited in the application see abstract see figure 2 ---	1-9
A	NUCLEIC ACIDS RESEARCH, vol. 18, no. 8, 25 April 1990, OXFORD, GB, page 2189 XP002007030 H. SHUKLA ET AL.: "The mRNA of a human class I gene HLA G7HLA 6.0 exhibits a restricted pattern of expression." see the whole document ---	1-9
A	EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 24, no. 1, January 1994, WEINHEIM, GERMANY, pages 176-180, XP002007031 M. ULBRECHT ET AL.: "HLA-G: expression in human keratinocytes in vitro and in human skin in vivo." see abstract see figure 2 ---	1-9
A	EP,A,0 412 700 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 13 February 1991 see examples see claims --- -/--	10-31

1

C.(Continuation) DOCUMENTS CO..SIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF IMMUNOLOGY, vol. 154, no. 8, 15 April 1995, BALTIMORE, MD, USA, pages 3771-3778, XP002007032 M. MCMASTER ET AL.: "Human placental HLA-G expression is restricted to differentiated cytotrophoblasts." see the whole document ---	1-31
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 92, no. 22, 24 October 1995, WASHINGTON, DC, USA, pages 10292-10296, XP002007033 A. BENSUSSAN ET AL.: "Detection of membrane-bound HLA-G translated products with a specific monoclonal antibody." see the whole document -----	1-31

INTERNATIONAL SEARCH REPORT

tional Application No
PCT/US 96/03765

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-412700	13-02-91	JP-A- 3155778	03-07-91

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